Inc*K* plasmid-mediated tetracycline resistance in *Edwardsiella ictaluri* isolates from diseased freshwater catfish in Vietnam

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ABSTRACT

Eight tetracycline resistant *Edwardsiella ictaluri* isolates obtained from diseased freshwater catfish (*Pangasianodon hypophthalmus)* in Vietnam, and showing different resistance phenotypes to other antimicrobial agents, were studied. The *tet* genes were determined using PCR. Conjugation experiments were performed to assess transferability of the tetracycline resistance determinant and the size and incompatibility group (Inc) of each *tet*-carrying plasmid were determined. PCR and sequencing were used for characterization of the co-transferred resistance genes. A *tetA* gene was demonstrated in the *E. ictaluri* isolates and for all of them, *E. coli* transconjugants were obtained. All transconjugants contained high-molecular weight *tetA*-carrying plasmids (~140 kb) belonging to the inc*K* group, as was shown with the PCR-based replicon typing method. The *strA-strB, dhfr1* and *sul 2* genes were detected on the *tetA*-carrying plasmids of the transconjugants showing resistance to streptomycin, trimethoprim and sulfonamides, respectively. The *dhfr1* gene was found to be located in a class 1 integron as determined by PCR and sequencing. Interestingly, the 3' CS region of class 1 integrons was not detected by PCR. This study shows the presence of inc*K* plasmid-mediated tetracycline resistance among *E. ictaluri* isolates from diseased freshwater catfish in Vietnam.

1. Introduction

Industrial aquaculture is a rapidly growing industry in many developed and developing countries, as Vietnam, where the freshwater catfish *Pangasianodon hypophthalmus* has grown into a global giant faster than any other aquaculture species in history. This indigenous fish species is high in demand from global consumers. With the rapid expansion and intensification of the freshwater industry, infectious diseases often break out. Bacillary necrosis caused by *Edwardsiella ictaluri*, is responsible for serious economical damage in Vietnamese catfish farms and for control of this disease, antimicrobial agents are often used both prophylatically and therapeutically (Crumlish et al., 2002; Ferguson et al., 2001). This may favor the spread of antimicrobial resistance genes in fish-associated and environmental aquatic bacteria.

In a recent study, acquired resistance to oxytetracycline was demonstrated in 52 of 64 Vietnamese *E. ictaluri* isolates tested and the majority of these isolates also showed acquired resistance to other antimicrobial agents, including streptomycin, sulphonamides and trimethoprim (Dung et al., 2008). Different mechanisms of resistance to tetracyclines have been described with ribosomal protection, efflux and enzymatic inactivation of the antibiotic as major modes of action. Most tetracycline resistant bacteria carry one or more of the 40 different tetracycline resistance genes described so far (Brown et al., 2008; Chopra et al., 2001; Robert, 2005).

The aim of the present study was to determine the genetic determinants of tetracycline resistance among *E. ictaluri* isolates from Vietnamese freshwater catfish and to assess its transferability. Genes encoding resistance to other antimicrobial agents which were co-transferred during conjugation experiments were also characterized.

2 Materials and methods

2.1 Bacterial isolates and determination of tet genes

Eight of the 52 tetracycline resistant *E. ictaluri* isolates obtained during a previous study and showing the three most prevalent antimicrobial resistance phenotypes (Dung et al., 2008), were selected (Table 1). All selected isolates were obtained from the kidney of diseased catfish (*Pangasianodon hypophthalmus)* during different outbreaks of bacillary necrosis in Vietnam. For the determination of the *tet* genes, PCR was performed (Cauwerts et al., 2006; Jun et al., 2004). Total DNA (genomic and plasmid DNA) and PCR mixtures were prepared as described previously (Baele et al., 2000; Martel et al., 2001).

2.2 Conjugation experiments

Conjugation experiments were carried out in Luria Broth medium with *E. coli* J5, resistant to rifampicin, used as the recipient strain. Tests were performed overnight at 37°C with a donor/recipient ratio of 0.2. Transconjugants were selected on MacConkey agar plates (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with tetracycline (25 mg/L) and rifampicin (250 mg/L) (Bertrand et al., 2006). The transfer frequency was estimated by dividing the number of transconjugants per milliliter by the number of recipients per milliliter.

2.3 Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *E. coli* transconjugants was determined by the Kirby Bauer disk diffusion test (Neo-sensitabs, Rosco Diagnostica, Taastrup, Denmark) as described previously

(document M31-A3) (Table 1) (NCCLS, 2008; Smet et al., 2008). Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (*E. coli* ATCC 25922).

2.4 Plasmid analysis

Plasmid profiles were determined for the *E. ictaluri* isolates and their *E. coli* transconjugants (Kado and Liu, 1981). The molecular size of each *tet*-carrying plasmid was estimated by using a BAC Tracker Supercoiled DNA ladder (Epicentre Biotechnologies, Madison, Wisconsin). Plasmid DNA of the *E. coli* transconjugants was obtained as described by Takahashi and Nagano (1984). The incompatibility (Inc) group of each *tet*-carrying plasmid was determined by the PCR-based replicon typing (PBRT) method (Carattoli et al., 2005).

2.5 Molecular characterization of co-transferred resistance genes

The characterization of the co-transferred resistance determinants on the *tet*-carrying plasmids were performed by PCR and sequencing on plasmid DNA of the *E. coli* transconjugants as described in previous reports (Bertrand et al., 2006; Costa et al., 2008; Huys et al., 2005; Schmidt et al., 2007; Zhang et al., 2004).

3. Results

PCR, with primers specific for different tetracycline resistance genes, demonstrated the presence of a *tetA* gene among all selected isolates.

E. coli transconjugants were obtained for all isolates. The characteristics of the *E. ictaluri* strains and their *tet*A-carrying plasmids are shown in Table 1. Transfer frequency was approximately 2.54×10^{-6}. Antimicrobial susceptibility testing of the *E. coli* transconjugants revealed that all other resistance determinants, with the exception of flumequine resistance, were cotransferred with the tetracycline resistance determinant. Plasmid analysis showed a strong band of approximately 140 kb indicating the presence of a high-molecular weight *tetA*-carrying plasmid for all transconjugants (data not shown). The PBRT method applied on plasmid DNA of the transconjugants showed that all plasmids carrying the *tetA* gene belonged to the inc*K* group.

Characterization of the co-transferred resistance determinants was performed by PCR on plasmid DNA with primers specific for trimethoprim, sulfonamide and streptomycin resistance genes. The *strA-strB* genes were detected on the *tetA*-carrying plasmids of the transconjugants that showed resistance to streptomycin. The *aadA* gene, another streptomycin resistance determinant, was not found. The *dhfr1* and *sul2* genes were found on the *tetA*-carrying plasmids of the transconjugants showing resistance to trimethoprim and sulphonamides, respectively. Often the trimethoprim resistance determinant is located in an integron. Therefore, PCR with primers specific for the class 1 (*intI1*) and class 2 (*intI2*) integrase was perfomed (Zhang et al., 2004)). Only the *intI1* gene was identified on all *tetA*-carrying plasmids indicating the presence of class 1 integrons. Characterization of the variable region of class 1 integrons by PCR and DNA sequencing revealed a 500 bp gene cassette, *dhfr1*. Class 1 integrons normally possess a 5' conserved segment (5'CS) and a 3' conserved segment (3'CS) separated by a variable region. However, the 3'CS, containing the *qacΔE1* and *sul1* genes and an open reading frame *orf5*, was not detected by PCR for all class 1 integrons located on the *tetA*-carrying plasmids.

4. Discussion

Recently, public health agencies have raised a worldwide concern about the impact of antimicrobial use in the aquaculture environment (Huys et al., 2005). The emergence of antimicrobial resistance among fish pathogens undermines the effectiveness of antimicrobial therapy in aquaculture. It also increases the possibilities for transfer of resistance determinants from aquatic bacteria to bacteria of terrestrial animals and human beings, including pathogens (Costa et al., 2008; Huys et al., 2005; Schmidt et al., 2001; Sun et al., 2009). Therefore, the exchange of antimicrobial resistance genes between bacteria in the aquaculture environment is of great concern. Very few information is available about plasmid-borne resistance genes among *E. ictaluri* isolates (Welch et al., 2009). Therefore, the genetic determinants of tetracycline resistance and its transferability were studied.

A *tetA* gene was demonstrated in the *E. ictaluri* isolates. This gene, as well as other tetracycline resistance determinants, has also been described in other fish pathogens (Aoki et al., 1987; Crumlish et al., 2002; Miranda et al., 2003; Schmidt et al., 2001; Sun et al., 2009). Several studies have investigated the genetic support of the *tetA* gene and found a Tn*1721*-like transposon to be involved in its mobility (Ojo et al., 2003; Rhodes et al., 2000; Sorum et al., 2003). The *tetA* gene present in our isolates may be carried by a Tn*1721*-like transposon, but this needs further investigation.

All transconjugants contained high-molecular weight *tetA*-carrying plasmids (~140 kb) belonging to the Inc*K* group, as was shown with the PBRT method. To our knowledge, this is the first description of the *tetA* gene on inc*K* plasmids in *E. ictaluri*. In a recent study an Inc*A/C* plasmid, containing genes encoding tetracycline resistance, was demonstrated in an *Edwarsiella ictaluri* strain (Welch et al., 2009). The *tetA* gene was also found to be located on a smaller plasmid in an *Aeromonas salmonicida*

isolate (Schmidt et al., 2001; Sørum et al., 2003). These findings might indicate that this gene is circulating among different plasmids. Further characterization of our inc*K* plasmids and comparison with other plasmids, containing tetracycline resistance determinants, may help to explain the spread of this gene among several plasmids.

The linked *strA-strB* gene pair was detected on the *tetA*-carrying plasmids of the transconjugants showing resistance to streptomycin. This gene pair is widely disseminated among diverse gram-negative bacteria and has also been detected in other bacteria isolated from farmed fish (L'Abée-Lund and Sørum, 2000; Sunde and Norström, 2005). A study in Norway characterized a small plasmid from the fish pathogen *Aeromonas salmonicida* and showed that the *strA-strB* genes were carried by a Tn*5393*-like transposon (L'Abée-Lund and Sørum, 2000). The genetic support of the linked *strA-strB* gene pair on our *tetA*-carrying plasmids remains unknown and needs further investigation.

The *dhfr1* gene, encoding resistance to trimethoprim, was found to be located in a class 1 integron as determined by PCR and sequencing. This gene cassette was also found in class 1 integrons associated with plasmids in clinical *Aeromonas salmonicida* isolates (Schmidt et al., 2001). Interestingly, the 3'CS was not detected in our class 1 integrons by PCR. This may indicate that the priming site in the 3'CS is missing. The presence of these 3'CS-lacking integrons has also been reported in bacteria from an aquatic environment and at low frequencies in *E. coli* recovered from humans and animals (Rosser and Young, 1999; Saenz et al., 2004; Vinué et al., 2008).

5. Conclusion

This study shows the presence of inc*K* plasmids carrying tetracycline, streptomycin, trimethoprim and sulphonamide resistance genes among *E. ictaluri* isolates from diseased freshwater catfish. It further strengthens the need for prudent use of antimicrobial agents in catfish production.

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Table 1

Characteristics of the *E. ictaluri* strains and the *tet*A-carrying plasmids analysed in this study

Isolate number	Year of isolation	Antimicrobial resistance[a] (parental strains)	co-transferred resistance	Antimicrobial resistance genes on the plasmid	Transfer frequency
E18	2002	TET, TMP	TMP	*tet*A, *dhfr*1	1.35×10^{-6}
E29	2002	TET, TMP	TMP	*tet*A, *dhfr*1	2.05×10^{-6}
LO2	2002	TET, TMP, SULF, STR	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	3.65×10^{-6}
QO2	2002	TET, TMP, SULF, STR	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	1.27×10^{-5}
198	2002	TET, TMP, SULF, STR, Flum	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	3.42×10^{-6}
192	2002	TET, TMP, SULF, STR, Flum	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	3.65×10^{-7}
E136	2005	TET, TMP, SULF, STR	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	1.03×10^{-7}
E137	2005	TET, TMP, SULF, STR	TMP, SULF, STR	*tet*A, *dhfr*1, *sul*2, *str*A-*str*B	3.90×10^{-6}

[a] Antimicrobial drugs used were the following: flumequine (Flum), tetracycline (TET), trimethoprim (TMP), streptomycin (STR), sulfonamides (SULF)